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(54) Title: A METHOD FOR DETECTION OF POTENTIAL CO-ANTIOXIDANTS

(57) Abstract

A method for screening and/or testing in vitro of synthetic or natural compounds for antioxidant potency. The use of 3,3',5,5'-tetra-*tert*-butyl-4,4'-bisphenol and pharmaceutical preparations thereof in restoring endothelial dysfunction.

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A METHOD FOR DETECTION OF POTENTIAL CO-ANTIOXIDANTS

Background

The method of the present invention is derived from a complex screening method (1) which relates to the oxidation and co-antioxidation of low density lipoprotein (LDL) lipids by a tocopherol-mediated mechanism (TMP, tocopherol-mediated peroxidation) (2). The latter mechanism summarizes a novel approach to explain the activity of α-tocopherol (α-TOH) in LDL in terms of both its ability to act as a phase transfer agent and also its role in the peroxidation of the lipid components of the lipoprotein. This earlier study (1) indicated that an effective co-antioxidant (XH) for α-TOH acted in three specific modes: the co-antioxidant must associate with an oxidizing LDL particle, reduce the lipid peroxidation chain carrying α-tocopheroxyl radical (α-TO•) [reaction 1], and the ensuing, co-antioxidant-derived radical (X•) must escape the lipoprotein particle (so as to minimize the possibility of regeneration of α-TO•)

$$XH + \alpha\text{-TO}\bullet \rightarrow \alpha\text{-TOH} + X\bullet \qquad [1]$$

The co-antioxidant efficacy was evaluated previously by the corresponding anti-TMP index (1), where anti-TMP indices of approximately 0 referred to highly effective co-antioxidants, and anti-TMP indices of approximately 100 indicated poor co-antioxidant activities. As this methodology required the isolation and labour-intensive work-up of biological material we sought to develop a simple and rapid screen to identify potential co-antioxidants for α-TOH, based upon the ability of a test compound to reduce α-TO• which was compartmentalized from the surrounding aqueous medium.

Description of tocopheroxyl radical attenuating ability (TRAA) test

The method of the present invention is based on the direct observation of α-TO• by electron paramagnetic resonance (EPR, a technique used specifically for the direct detection of free radical species) spectroscopy and the subsequent effect of the addition of a potential co-antioxidant which will reduce this radical. In brief, the method we have developed makes use of α-TO• generated in micellular suspensions of α-TOH using a commercially available mercury-vapour lamp. The α-TO• generated under the test conditions was found to decay with a half life of approximately 25 min in the absence of an added co-antioxidant. Measurement of the observed rate of decay of α-TO• in both the absence and presence of a potential co-antioxidant permitted the determination of a relative rate index which then allowed the comparison of the tocopheroxyl radical attenuating ability (TRAA) of each test compound to be determined. We defined an efficient inhibitor of the α-TO• (i.e. co-antioxidant) as a compound which caused a greater than 5-fold increase in the rate of decay of the α-TO• radical as compared with the rate in the absence of the test compound.

Thus, the present invention provides a method for screening and/or testing in vitro synthetic or natural compounds for co-antioxidant potency in eliminating the α-tocopheryl radical, characterised by measuring the decay of this radical in the presence or absence of the test compound using EPR spectroscopy and identifying co-antioxidants which exhibit a greater than 5-fold increase in the rate of decay of the α-tocopheryl radical.

Details of the TRAA test

Preparation of micelles --- 100 mM Stock solutions of cetyltrimethyl ammonium chloride (HTAC) and sodium dodecyl sulfate (SDS, both obtained from Aldrich) were prepared in phosphate buffer principally as described in (3). Micellular dispersions of α-TOH were prepared by diluting an ethanolic solution of α-TOH (0.2 M) into such micelles (3) at a final α-TOH concentration of 500 μM. This resulting solution was sonicated (30 W, operating frequency 41±6 kHz) for 15 seconds after which time it was completely homogenous.

Generation and detection of a-TO•--- Aliquots of the α-TOH-containing micelles were placed into the neck of an EPR flat cell (100 μL, Wilmad Glass Co., Buena, NJ) and placed 0.5 m from a 125 W Osram HOL-Mercury fluorescent bulb (GEC distributors, Roseberry, Sydney) used as a UV-light source. To increase the light intensity, the frosted casing of the bulb was removed. Samples were irradiated for 3 min, followed by thorough mixing and subsequent transfer of the flat cell to the corresponding temperature controlled Dewar insert (Wilmad) in the EPR cavity, where the sample was allowed to equilibrate to 37°C. This procedure afforded α-TO• levels between 1-2 μM as estimated against a nitroxide standard. Unless specified otherwise EPR spectra were obtained at 9.41 GHz with modulation amplitude 1.0 G, microwave power 20 mW, and modulation frequency 12.5 kHz using a Bruker ESP 300 EPR spectrometer fitted with an X-band cavity. Temperature control was obtained using a Bruker Temperature Control Unit and temperatures were accurate to ± 0.5°C.

Determination of TRAA--- Following accumulation of the 'T=O min' spectrum, the flat cell arrangement containing α-TO• was removed from the EPR cavity and the solution gently coaxed into the neck of the flat cell under positive pressure. The compound to be tested (or the appropriate volume of water or ethanol for the controls) was then added to give a final concentration of 10 μM and the treated sample replaced in the cavity, allowed to equilibrate to standard conditions and sampling resumed. This method of addition normally required 3 min in total, and did not affect the rate of decay of α-TO• as verified by identical decays in control samples treated in the same fashion with or without an appropriate volume ethanol. The time-dependant decay of the EPR signal intensity for α-TO• was measured in both the presence and absence of the test co-antioxidant (10 μM) using a sweep time of 20.5 s, averaging the output from 3 successive sweeps at each time point, and averaging the results of 3 separate experiments. Control decay curves (i.e. in the absence of the co-antioxidant) were run periodically between separate experiments and averaged over the sample set to afford observed rate constants in the range $k_1=4.6\pm0.3 \times 10^{-4}$s. A relative decay term $k_{(+antioxidant)}/k_{(-antioxidant)}$ was then obtained and compared with the definition of an effecient inhibitor of α-TO•, i.e. caused a greater then 5-fold increase in the rate of decay of the radical.

Results

The method developed was applied to 63 different natural and synthetic compounds. To establish the reliability of the method, we compared the TRAA of these 63 potential co-antioxidants with their respective anti-TMP activities, i.e., their ability to inhibit the early stages of LDL lipid peroxidation initiated by a low flux of water-soluble, peroxyl radicals. The relationship between the measured TRAA and corresponding anti-TMP activity was highly significant ($p < 0.00005$, Rank test), so that the efficiency of a co-antioxidant for LDLs α-TOH could be predicted with > 93% probability by the TRAA test alone.

Strikingly, probucol [4,4'-(isopropylidenedithio)*bis*(2,6-di-*tert*-butylphenol)] showed low activity in both the TRAA ($k_{(+)}/k_{(-)}=0.92$) and anti-TMP test (anti-TMP index of 98), even though this compound is known to inhibit both LDL oxidation under more severe oxidizing conditions and atherosclerosis in several animal models. In contrast, the probucol metabolite 3,3',5,5'-tetra-*tert*-butyl-4,4'-bisphenol showed high activity in the TRAA test (i.e., immediate decay of α-TO• upon addition of the compound), and this correlated with its high anti-TMP activity (anti-TMP index of 8.0). From these results, we predicted that the probucol metabolite, rather than probucol itself, is the active antioxidant inhibiting LDL oxidation and atherosclerosis. Consistent with this, the probucol metabolite is detected in LDL of animals supplemented with probucol, and the concentration of the metabolite detected ex vivo is sufficient to provide high activity in the TRAA and anti-TMP tests.

Accumulation of lipids in vessel walls and subsequent lipid peroxidation is considered to be an early event in atherogenesis. This development is also often accompanied by a decreased endothelial function, characterized by a reduced ability for the natural stimulus induced relaxation of the smooth muscles of the vessel and sometimes even resulting in an abnormal contraction.

Thus, a further aspect of the present invention relates to the use of 3,3',5,5'-tetra-*tert*-butyl-4,4'-bisphenol or a physiologically acceptable salt thereof for the manufacture of a medicament with effect in inhibiting lipid peroxidation, particularly for restoring endothelial dysfunction.

A still further aspect of the present invention relates to a pharmaceutical preparation for use in the prophylaxis and/or treatment of conditions of oxidative stress where restoring endothelial dysfunction is essential, in which preparation the active ingredient is 3,3',5,5'-tetra-*tert*-butyl-4,4'-bisphenol or a physiologically acceptable salt thereof.

A still further aspect of the present invention relates to a method of restoring endothelial dysfunction in mammals, including man, wherein an effective amount of 3,3',5,5'-tetra-*tert*-butyl-4,4'-bisphenol or a physiologically acceptable salt thereof is administered to a host in need of such treatment.

The dosage of 3,3',5,5'-tetra-*tert*-butyl-4,4'-bisphenol is suitably in the range 1-1000 mg/day.

General application of the TRAA test

The method of this invention provides a simple and rapid in vitro test for the screening and/or testing of synthetic and natural compounds for their co-antioxidant potency for α-TOH. While designed specifically for the screening of compounds for their anti-TMP activity in LDL in light of potential anti-atherosclerotic activity, the test generally identifies any compound that can reduce and hence interact with phenoxyl radicals in (protein-containing) lipid emulsions or emulsion-like fluids (including lipoproteins, lipid emulsions used in parenteral and other nutrition, and oils). As such, the method can be applied to the screening and/or testing of inhibitors of lipid oxidation in each of the above systems, whether applied to a medical or technical field. In the area of medicine, the test described is generally useful for the identification of drugs that act at least in part by inhibiting or preventing lipid oxidation under oxidative stress or conditions where oxidative stress is implied, such as ageing, inflammation, neurological disorders, ischaemia/reperfusion, and cardiovascular disease.

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(1) Bowry, V.W., D. Mohr, J. Cleary, and R. Stocker. 1995. Prevention of tocopherol-mediated peroxidation of ubiquinol-10-free human low density lipoprotein. *J. Biol. Chem.* 270; 5756-5763.

(2) Bowry, V.W., and R. Stocker. 1993. Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J. Am. Chem. Soc.* 115: 6029-6040.

(3) Bisby, R.H. and A. W. Parker. 1991. Reactions of the alpha-tocopheroxyl radical in micellar solutions studied by nanosecond laser flash photolysis. *FEBS Lett. 290: 205-208.*

CLAIMS

1. A method for screening and/or testing in vitro synthetic or natural compounds for co-antioxidant potency in eliminating the α-tocopheryl radical, characterised by measuring the decay of this radical in the presence or absence of the test compound using electron paramagnetic resonance spectroscopy and identifying co-antioxidants which exhibit a greater than 5-fold increase in the rate of decay of the α-tocopheryl radical.

2. A method as claimed in claim 1 wherein the said radicals are present in micellular suspensions.

3. A method as claimed in claim 1 or claim 2 wherein the said radicals are generated by use of a mercury-vapor lamp.

4. A method as claimed in any one of the preceding claims wherein the co-antioxidant which is identified as passing the 5-fold increase requirement is 3,3', 5,5'-tetra-*tert*-butyl-4,4'-bisphenol.

5. A pharmaceutical preparation for use in the prophylaxis and/or treatment of oxidative stress where the prevention of lipid oxidation is required characterised in that a co-antioxidant as defined in claim 1 is incorporated with an excipient carrier or diluent to form the pharmaceutical preparation.

6. A pharmaceutical preparation according to claim 5 wherein the preparation is in unit dosage form.

7. A pharmaceutical preparation as claimed in claim 5 or claim 6 comprising the co-antioxidant wherein the pharmaceutically accepted carrier is a synthetic and/or natural additional co-antioxidant.

8. A pharmaceutical preparation as claimed in any one of claims 5 to 7 wherein the co-antioxidant is 3,3', 5,5'-tetra-*tert*-butyl-4,4'-bisphenol.

9. The use of 3,3',5,5'-tetra-*tert*-butyl-4,4'-bispenol or a physiologically acceptable salt thereof for the manufacture of a medicament with effect in inhibiting lipid peroxidation.

10. The use of 3,3', 5,5'-tetra-*tert*-butyl-4,4'-bisphenol or a physiologically acceptable salt thereof for the manufacture of a medicament with effect in restoring endothelial dysfunction.

11. A method for inhibiting lipid peroxidation in mammals, including man, wherein an effective amount of a compound which has been identified by the method claimed in any one of claims 1 to 4 meet the greater than 5-fold increase test is administered to a host in need of such treatment.

12. A method for restoring endothelial dysfunction in mammals, including man, wherein an effective amount of the compound 3,3', 5,5'-tetra-*tert*-butyl-4,4'-bisphenol is administered to a host in need of such treatment.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/00575

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 31/05, G01R 33/60
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, G01R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochimica et Biophysica Acta, Volume 801, 1984, Marina Scarpa et al, "Formation of alpha-Tocopherol Radical and Recycling of alpha-Tocopherol by Ascorbate during Peroxidation of Phosphatidylcholine Liposomes" page 215 - page 219	1-3
A	--	4
X	US 4115590 A (SIDNEY I. LERNER), 19 Sept 1978 (19.09.78)	5-10
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☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search	Date of mailing of the international search report
27 June 1997	24 -07- 1997
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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/00575

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 11-12
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/06/97

International application No.
PCT/SE 97/00575

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4115590 A	19/09/78	NONE	